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BIOLOGY AND GENETICS OF PRION DISEASES

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CONTENTS

ABSTRACT

Enriching fractions from Syrian hamster (SHa) brain for scrapie prion infectivity led to the discovery of the prion protein (PrP). Prion diseases include scrapie of sheep, bovine spongiform encephalopathy (BSE) of cattle, as well

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as Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), and fatal familial insomnia (FFI) of humans. Discovery of mutations in the PrP genes of humans with familial CJD, GSS, and FFI established that prion diseases are both genetic and infectious. Many lines of evidence have converged to argue that infectious prions are composed largely, if not entirely, of PrP^{Sc} molecules. Mice overexpressing mutant and wild-type transgenes develop neurologic illnesses spontaneously and produce prions as demonstrated by serial transmission of disease in rodents after inoculation of brain extracts. Although these and many other findings argue that prions are devoid of nucleic acid, the molecular basis of prion strains remains enigmatic. The formation of PrP^{Sc} from PrP^{C} is a posttranslational process involving the conversion of α-helices into β-sheets. This conformational change in PrP appears to be the fundamental event that underlies prion propagation and the pathogenesis of prion diseases. The unique features of prion structure and propagation differentiate prions from all other transmissible pathogens.

INTRODUCTION

A set of remarkable discoveries in the past three decades has led to the molecular and genetic characterization of the transmissible pathogen causing scrapie in animals and a quartet of illnesses in humans: kuru, CJD, GSS, and FFI (Table 1). To distinguish this infectious pathogen from viruses and viroids, the term *prion* was introduced to emphasize its proteinaceous and infectious nature (146). An abnormal isoform of the prion protein (PrP), PrP^{Sc}, is the only known component of the prion (148). PrP is encoded by a gene on the short arm of chromosome 20 in humans (173). PrP^{Sc} differs physically from the normal, cellular isoform PrP^{C} by its high β-sheet content, its insolubility in detergents, its propensity to aggregate, and its relative resistance to proteolysis (131, 134, 138).

Accumulation of PrP^{Sc} in the brain has been observed with most of the human prion diseases. The presence of PrP^{Sc} implicates prions in the pathogenesis of these diseases. However, in rare patients (23, 126) and some transgenic (Tg) mice that appear to have low or undetectable amounts of PrP^{Sc}, neurodegeneration seems, at least in part, to be caused by abnormal metabolism of mutant PrP (93). The usefulness of distinguishing between prion diseases in which transmission can and cannot be demonstrated with current animal models remains to be established (153). As our knowledge of the prion diseases increases and more is learned about the molecular and genetic characteristics of prion proteins, we will undoubtedly reclassify these disorders. Indeed, the discovery of PrP and the identification of pathogenic PrP gene mutations have already forced us to view these illnesses from perspectives not previously imagined.

Table 1 Human prion diseases

Disease	Etiology
Kuru	Infection
Creutzfeldt-Jakob disease	
Iatrogenic	Infection
Sporadic	Unknown
Familial	PrP mutation
Gerstmann-Sträussler-Scheinker disease	PrP mutation
Fatal familial insomnia	PrP mutation

DEVELOPMENT OF THE PRION CONCEPT

The scrapie literature contains a fascinating record of all the structural hypotheses proposed to explain the physicochemical structure of the infectious particles. Among the earliest hypotheses was the notion that scrapie was a disease of muscle caused by the parasite *Sarcosporidia* (131a, 131b). With the successful transmission of scrapie to animals, the hypothesis that scrapie is caused by a filterable virus became popular (40, 191). When Tikvah Alper and her colleagues discovered that scrapie infectivity resists inactivation by UV and ionizing radiation (3, 4), a myriad of hypotheses on the chemical nature of the scrapie agent emerged. The resistance of scrapie infectivity to inactivation by UV and ionizing radiation led to the proposal that the scrapie agent might be a naked nucleic acid similar to plant viroids (52), but subsequent studies disproved that suggestion (53). The term unconventional virus was proposed, but no structural details were ever given with respect to how these unconventional virions differ from conventional viral particles (69). Some have thought that this term, unconventional, reflected the ignorance surrounding the structure of the infectious particle causing scrapie (140).

Once an effective protocol was developed for preparation of partially purified fractions of scrapie agent from hamster brain, investigators could demonstrate that procedures modifying or hydrolyzing proteins diminish scrapie infectivity (146, 155). At the same time, tests done in search of a scrapie-specific nucleic acid could not demonstrate any dependence of infectivity on a polynucleotide (146), in agreement with earlier studies reporting the extreme resistance of infectivity to UV irradiation at 254 nm (3).

Based on these findings, the term prion was introduced to distinguish the proteinaceous infectious particles that cause scrapie, CJD, GSS, and kuru from both viroids and viruses (146). Hypotheses for the structure of the infectious prion particle included: (*a*) proteins surround a nucleic acid that encodes them

(a virus), (*b*) proteins are associated with a small polynucleotide, and (*c*) proteins are devoid of nucleic acid (146). Mechanisms postulated for the replication of infectious prion particles ranged from those used by viruses to the synthesis of polypeptides in the absence of a nucleic acid template to posttranslational modifications of cellular proteins. Subsequent discoveries have narrowed the hypotheses for both prion structure and the mechanism of replication.

Considerable evidence has accumulated over the past decade supporting the prion hypothesis (148). Furthermore, the replication of prions and their mode of pathogenesis also appear to be without precedent. After a decade of severe criticism and serious doubt, the prion concept is now enjoying considerable acceptance.

DISCOVERY OF THE PRION PROTEIN

After it was established that scrapie prion infectivity in partially purified fractions depended upon protein (155), the search for a scrapie-specific protein intensified. While the insolubility of scrapie infectivity made purification problematic, this property and the relative resistance to degradation by proteases were used to extend the degree of purification. Radioiodination of partially purified fractions revealed a protein unique to preparations from scrapie-infected brains (16, 150). This molecule was later named prion protein and abbreviated PrP. The protease-resistant core of PrP has an M_r of 27–30 kDa; it is also known as PrP 27–30 (122). Analyses by others rapidly confirmed its existence (54).

Subsequent studies showed that PrP 27–30 is derived from a larger protein of M_r 33–35 kDa, which is designated PrP^{Sc} (131, 134). At the same time, the brains of normal and scrapie-infected hamsters were found to express similar levels of PrP mRNA and a protease-sensitive prion protein designated PrP^{C} (134). PrP^{C} or a subset of PrP molecules is the substrate for PrP^{Sc}. Many lines of evidence argue that PrP^{Sc} is an essential component of the infectious prion particle:

1. PrP 27–30 and scrapie infectivity copurify by biochemical methods. Concentration of PrP 27–30 is proportional to prion titer (16, 88, 97, 122, 150, 165, 183).
2. The kinetics of proteolytic digestion of PrP 27–30 and infectivity are similar (16, 122, 150).
3. Immunoaffinity chromatography has shown copurification of PrP^{Sc} and infectivity. Also, α-PrP antisera neutralizes infectivity (64, 66).
4. PrP^{Sc} is detected only in clones of cultured cells producing infectivity (29, 124, 180).

5. PrP amyloid plaques are specific for prion diseases of animals and humans (10, 46, 106, 160). Deposition of PrP amyloid is controlled, at least in part, by the PrP sequence (156).
6. PrP^{Sc} (or PrP^{CJD}) is specific for prion diseases of animals and humans (14, 22, 170).
7. MoPrP gene and scrapie incubation times are genetically linked (30, 31, 94, 157). The PrP gene harbored by mice with long incubation times encodes amino acid substitutions at codons 108 and 189, as compared with mice with short or intermediate incubation times (187).
8. The level of SHaPrP transgene expression and the primary structure of PrP^{Sc} in the inoculum governs the species barrier, scrapie incubation times, neuropathology, and prion synthesis in mice (156, 167).
9. PrP gene point mutations at codons 102, 178, 198, or 200 are genetically linked to the development of inherited prion diseases in humans (55, 67, 89, 142). Genetic linkage was also established between the mutation insert of six additional octarepeats and familial CJD (144).
10. Mice expressing MoPrP transgenes with the point mutation of GSS spontaneously develop neurologic dysfunction, spongiform brain degeneration, and astrocytic gliosis (93).
11. Ablation of the PrP gene in mice prevents scrapie and propagation of prions after intracerebral inoculation of prions (27, 152).
12. Mice expressing chimeric Mo/SHaPrP transgenes produce "artificial" prions with novel properties (168).

So far, all attempts to find a second component of the prion particle have been unsuccessful.

Although some investigators contend that PrP^{Sc} is merely a pathologic product of scrapie infection and that this molecule coincidentally purifies with the scrapie virus (1, 2, 20, 171, 172), few data support this view. No infective fractions containing <1 PrP^{Sc} molecule per ID_{50} unit have been found; such a result would indicate that PrP^{Sc} is not required for infectivity. Some investigators report that PrP^{Sc} accumulation in hamsters occurs after the synthesis of many infective units (41, 42, 166), but these results have been refuted (97). The discrepancy appears to result from comparisons of infectivity in crude homogenates with PrP^{Sc} concentrations measured in purified fractions. In another study, the investigators claimed to have dissociated scrapie infectivity from PrP 27–30 in brains of Syrian hamsters treated with amphotericin B and inoculated with the 263K isolate but not from animals inoculated with the 139H isolate; also, no dissociation was seen with mice inoculated with Me7 prions (192).

The discovery of PrP 27–30 in fractions enriched for scrapie infectivity was accompanied by the identification of rod-shaped particles (150, 154). The rods

are ultrastructurally indistinguishable from many purified amyloids and display the tinctorial properties of amyloids (154). The formation of prion rods requires limited proteolysis in the presence of detergent (123). Thus, the prion rods in fractions enriched for scrapie infectivity are largely, if not entirely, artifacts of the purification protocol. Solubilization of PrP 27–30 into liposomes with retention of infectivity (65) demonstrated that large PrP polymers are not required for infectivity and permitted the immunoaffinity copurification of PrP^{Sc} and infectivity (64, 66). Of note, the amyloid plaques in prion diseases contain PrP, as determined by immunoreactivity and amino acid sequencing (10, 46, 106, 160, 178). Some investigators believe that scrapie-associated fibrils are synonymous with the prion rods and are composed of PrP even though these fibrils can be distinguished ultrastructurally and tinctorially from amyloid polymers (128, 129).

PrP GENE STRUCTURE, ORGANIZATION, AND EXPRESSION

The entire open reading frame (ORF) of all known mammalian and avian PrP genes resides within a single exon (8, 68, 89, 187). This feature of the PrP gene eliminates the possibility that PrP^{Sc} arises from alternative RNA splicing (8, 187, 188). The two exons of the Syrian hamster (SHa) PrP gene are separated by a 10-kb intron: exon 1 encodes a portion of the 5′ untranslated leader sequence while exon 2 encodes the ORF and 3′ untranslated region (8). The mouse (Mo) and sheep PrP genes contain three exons with exon 3 analogous to exon 2 of the hamster (188, 248, 249). The promoters of both the SHa and MoPrP genes contain multiple copies of G-C rich repeats and are devoid of TATA boxes. These G-C nonamers represent a motif that may function as a canonical binding site for the transcription factor Sp1 (125).

That PrP genes can be mapped to the short arm of human chromosome 20 and the homologous region of Mo chromosome 2 argues for the existence of PrP genes prior to the speciation of mammals (173). Hybridization studies demonstrated <0.002 PrP gene sequences per ID_{50} unit in purified prion fractions, indicating that a gene encoding PrP^{Sc} is not a component of the infectious prion particle (134). This is a major feature that distinguishes prions from viruses including those retroviruses that carry cellular oncogenes and from satellite viruses that derive their coat proteins from other viruses previously infecting plant cells. Purified fractions enriched for prion infectivity were analyzed for a scrapie-specific nucleic acid using a specially developed technique designated return refocusing gel electrophoresis, but no such nucleic acid was found (130). These studies argue that if such a molecule exists, then its size is 80 nt or less (101, 158).

Although PrP mRNA is constitutively expressed in the brains of adult animals (36, 134), it is highly regulated during development. In the septum, levels of PrP mRNA and choline acetyltransferase increase in parallel during development (132). In other brain regions, PrP gene expression occurs at an earlier age. In situ hybridization studies show that the highest levels of PrP mRNA occur in neurons (110).

EXPERIMENTAL SCRAPIE

For many years, studies of experimental scrapie were performed exclusively with sheep and goats. The disease was first transmitted by intraocular inoculation (40) and later by intracerebral, oral, subcutaneous, intramuscular, and intravenous injections of brain extracts from sheep developing scrapie. Incubation periods of 1–3 years were common and often many of the inoculated animals failed to develop disease (51, 83, 84). Different breeds of sheep exhibited markedly different susceptibilities to scrapie prions inoculated subcutaneously, suggesting that the genetic background might influence host permissiveness (82).

Studies of PrP genes (*Prn-p*) in mice with short and long scrapie incubation times demonstrated genetic linkage between a *Prn-p* restriction fragment length polymorphism (RFLP) and a gene modulating incubation times (*Prn-i*) (31). Other investigators have confirmed the genetic linkage, and one group has shown that the incubation time gene *Sinc* is also linked to PrP (94, 157). *Sinc* was first described by Dickinson and colleagues over 25 years ago (49), but the term is no longer used (133a). Whether the genes for PrP and *Prn-i* are all congruent remains to be established. The PrP sequences of NZW ($Prn\text{-}p^a$) and I/Ln ($Prn\text{-}p^b$) mice with short and long scrapie incubation times, respectively, differ at codons 108 (L→F) and 189 (T→V) (187). Although these amino acid substitutions argue for the congruency of *Prn-p* and *Prn-i*, experiments with $Prn\text{-}p^a$ mice expressing $Prn\text{-}p^b$ transgenes demonstrated a paradoxical shortening of incubation times (188) instead of a prolongation as predicted from ($Prn\text{-}p^a \times Prn\text{-}p^b$) F_1 mice, which exhibit long incubation times. We described those findings as "paradoxical shortening" because we and others had believed for many years that long incubation times are dominant traits (31, 49). From studies of congenic and transgenic mice expressing different numbers of the *a* and *b* alleles of *Prn-p*, we now realize that these findings were not paradoxical; indeed, they result from increased PrP gene dosage (29a).

HUMAN PRION DISEASES

The human prion diseases manifest as infectious, inherited, and sporadic disorders and are often referred to as kuru, CJD, GSS, and FFI, depending upon the

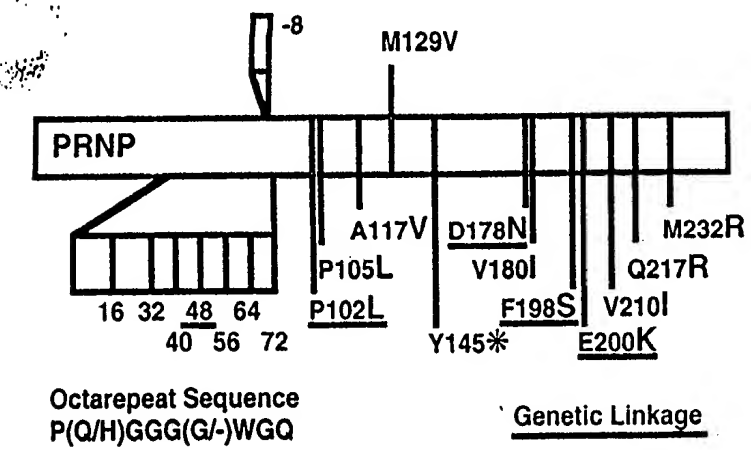

Figure 1 Human prion protein gene (PRNP). The large gray rectangle denotes the open reading frame (ORF). Human PRNP wild-type polymorphisms appear above the rectangle while mutations that segregate with the inherited prion diseases are below. The wild-type human PrP gene contains five octarepeats [P(Q/H)GGG(G/-)WGQ] from codons 51 to 91. Deletion of a single octarepeat at codon 81 or 82 is not associated with prion disease. Common polymorphisms occur at codons 117 (Ala → Ala) and 129 (Met→Val); homozygosity for Met or Val at codon 129 appears to increase susceptibility to sporadic CJD. Octarepeat inserts of 16, 32, 40, 48, 56, 64, and 72 amino acids at codons 67, 75, or 83 are designated by the small rectangle below the ORF. These inserts segregate with familial CJD, and significant genetic linkage has been demonstrated where sufficient specimens from family members are available. Point mutations are designated by the wild-type amino acid preceding the codon number, and the mutant residue follows, e.g. P102L. These point mutations segregate with the inherited prion diseases, and significant genetic linkage (underlined mutations) has been demonstrated where sufficient specimens from family members are available. (Reprinted, with permission, from Ref. 149.)

clinical and neuropathological findings (Table 1). Infectious forms of prion diseases result from the horizontal transmission of the infectious prions, as occurs in iatrogenic CJD and kuru. Inherited forms, notably GSS, familial CJD, and FFI, comprise 10–15% of all cases of prion disease. A mutation in the ORF or protein-coding region of the PrP gene has been found in all reported kindreds with inherited human prion disease. Sporadic forms of prion disease comprise most cases of CJD and possibly some cases of GSS (121). How prions arise in patients with sporadic forms is unknown, but hypotheses include horizontal transmission from humans or animals (69), and somatic mutation of the PrP gene ORF and spontaneous conversion of PrP^{C} into PrP^{Sc} (91, 147). Numerous attempts to establish an infectious link between sporadic CJD and a preexisting prion disease in animals or humans have been unrewarding (39, 86, 115).

The recognition that ~10% of CJD cases are familial led to the suspicion that genetics plays a role in this disease (43, 63, 96, 118–120, 127, 163, 177);

in addition, most cases of GSS are familial (72). Like sheep scrapie, the relative contributions of genetic and infectious etiologies in the human prion diseases remained puzzling.

The discovery of the PrP gene and its linkage to scrapie incubation times in mice (31) raised the possibility that mutation might feature in the hereditary human prion diseases. A proline (P)→leucine (L) mutation at codon 102 was shown to be linked genetically to development of GSS with a LOD (logarithm of the odds) score exceeding 3 (Figure 1) (89). This mutation may be caused by the deamination of a methylated CpG in a germline PrP gene resulting in the substitution of a thymine (T) for cytosine (C). The P102L mutation has been found in ten different families in nine different countries, including the original GSS family (56, 81, 108, 109).

An insert of 144 bp containing six octarepeats at codon 53 was described in patients with CJD from four families residing in southern England (Figure 1) (37, 136, 144). This mutation must have arisen through a complex series of events because the human PrP gene contains only five octarepeats, indicating that a single recombination event could not have created the insert. Genealogic investigations have shown that all four families are related, arguing for a single founder born more than two centuries ago. The LOD score for this extended pedigree exceeds 11. Studies from several laboratories have demonstrated that two, four, five, six, seven, eight, or nine octarepeats in addition to the normal five are found in individuals with inherited CJD (21, 76, 135, 136), whereas deletion of one octarepeat has been identified without the neurologic disease (111, 137, 184).

For many years the unusually high incidence of CJD among Israeli Jews of Libyan origin was thought to result from the consumption of lightly cooked sheep brain or eyeballs (99). Recent studies have shown that some Libyan and Tunisian Jews in families with CJD have a PrP gene point mutation at codon 200 that produces a glutamate (E)→lysine (K) substitution (79, 91). The E200K mutation has also been found in Slovaks originating from Orava in north-central Czechoslovakia (79), in a cluster of familial cases in Chile (77), and in a large German family living in the United States (11).

Many families with CJD have a point mutation at codon 178 resulting in an aspartic acid (D)→asparagine (N) substitution (59, 78). Recently, a new prion disease that presents with insomnia has been described in three Italian families with the D178N mutation (112, 126). The neuropathology in these patients with FFI is restricted to selected nuclei of the thalamus. Whether all patients with the D178N mutation or only a subset present with sleep disturbances is unclear. It has been proposed that the allele with the D178N mutation encodes a methionine at position 129 in FFI while a valine is encoded at position 129 in familial CJD (80). The discovery that FFI is an inherited prion disease clearly widens the clinical spectrum of these disorders and raises the

possibility that many other degenerative diseases of unknown etiology may be caused by prions (98, 126).

A valine (V)→isoleucine (I) mutation at PrP codon 210 produces CJD with classic symptoms and signs (143, 159). Apparently, this V210I mutation is also incompletely penetrant.

Other point mutations at codons 105, 117, 145, 198, 217, and possibly 232 also segregate with inherited prion diseases (21, 56, 90, 92, 104, 105, 182). Patients with a dementing or telencephalic form of GSS have a mutation at codon 117. These patients as well as some in other families were once thought to have familial Alzheimer's disease but are now known to have prion diseases on the basis of PrP immunostaining of amyloid plaques and PrP gene mutations (58, 73, 74, 133). Patients with the codon 198 mutation have numerous neurofibrillary tangles that stain with antibodies to τ and have amyloid plaques (58, 73, 74. 133) that are composed largely of a PrP fragment extending from residues 58 to 150 (178). A genetic linkage study of this family produced a LOD score exceeding 6 (55). The neuropathology of two patients of Swedish ancestry with the codon 217 mutation (95) was similar to that of patients with the codon 198 mutation.

Some GSS patients have a leucine substituted for proline at PrP codon 105 (105). In one patient with a prolonged neurologic illness spanning almost two decades, PrP amyloid plaques showed that the patient had an amber mutation of the PrP gene resulting in a stop codon at residue 145 (104). Staining the plaques with α-PrP peptide antisera suggested that the plaques might be composed exclusively of the truncated PrP molecules. That a PrP peptide ending at residue 145 would polymerize in amyloid filaments is not surprising because an earlier study, noted above, showed that the major PrP peptide in plaques from patients with the F198S mutation was an 11-kDa PrP peptide beginning at codon 58 and ending at ~150 (178). Furthermore, synthetic PrP peptides adjacent to and including residues 109 to 122 readily polymerize into rod-shaped structures with the tinctorial properties of amyloid (38, 60, 71, 75).

Iatrogenic Creutzfeldt-Jakob Disease

Accidental transmission of CJD to humans appears to have occurred with corneal transplantation (213), contaminated EEG electrode implantation (199), and surgical operations using contaminated instruments or apparatuses (Table 2) (211, 222, 227, 245). A cornea unknowingly removed from a donor with CJD was transplanted to an apparently healthy recipient, who developed CJD after a prolonged incubation period. Corneas of animals have significant levels of prions (206), making this scenario seem quite probable. The same improperly decontaminated EEG electrodes that caused CJD in two young patients

Table 2 Infectious prion diseases of humans—iatrogenic Creutzfeldt-Jakob disease

Source	No. cases
1. Depth electrodes	2
2. Corneal transplants	1
3. Human pituitary growth hormone	55
4. Human pituitary gonadotropin	5
5. Dura mater grafts	11
6. Neurosurgical procedures	4
Total	78

with intractable epilepsy were found to cause CJD in a chimpanzee 18 months after their experimental implantation (200).

Surgical procedures may have resulted in accidental inoculation of patients with prions during their operations (69, 204, 245), presumably because some instrument or apparatus in the operating theater became contaminated when a CJD patient underwent surgery. Although the epidemiology of these studies is highly suggestive, no proof for such episodes exists.

Since 1988, eleven cases of CJD after implantation of dura mater grafts have been recorded (204, 225, 228, 229, 231, 232, 243, 246). All of the grafts were thought to have been acquired from a single manufacturer whose preparative procedures were inadequate to inactivate human prions (204). One case of CJD occurred after repair of an eardrum perforation with a pericardium graft (241).

Thirty cases of CJD in physicians and health care workers have been reported (198); however, no occupational link has been established (240). Whether any of these cases represent infectious prion diseases contracted during care of patients with CJD or processing specimens from these patients remains uncertain.

Human Growth Hormone Therapy

The possibility of transmission of CJD from contaminated human growth hormone (HGH) preparations derived from human pituitaries has been raised by the occurrence of fatal cerebellar disorders with dementia in >55 patients ranging in age from 10 to 41 years (Table 2) (202–205, 215). While one case of spontaneous CJD in a 20-year-old woman has been reported (202, 218, 233), CJD in patients under 40 years of age is very rare. These patients received injections of HGH every 2 to 4 days for 4 to 12 years (195, 201, 210, 214, 218, 221, 224, 226, 230, 236, 244). Interestingly, most of the patients presented with cerebellar syndromes that progressed over periods varying from 6 to 18 months. Some patients became demented during the terminal phase of their

illnesses. The clinical courses of some patients with dementia occurring late resemble kuru more than ataxic CJD in some respects (237). Assuming these patients developed CJD from injections of prion-contaminated HGH preparations, the possible incubation periods range from 4 to 30 years (204). Incubation periods of two to three decades have been suggested to explain cases of kuru in recent years (216, 220, 237). Many patients received several common lots of HGH at various times during their prolonged therapies, but no single lot was administered to all the American patients. An aliquot of one lot of HGH has been reported to transmit CNS disease to a squirrel monkey after a prolonged incubation period (217). How many lots of the HGH might have been contaminated with prions is unknown.

Although CJD is a rare disease with an incidence of approximately one per million population (227), it is reasonable to assume that CJD occurred with a proportional frequency among dead people. About 1% of the population dies each year and most CJD patients die within one year of developing symptoms. Thus, we estimate that 1 per 10^4 dead people had CJD. Since 10,000 human pituitaries are typically processed in a single HGH preparation, the possibility of hormone preparations contaminated with CJD prions is not remote. The concentration of CJD prions within infected human pituitaries is unknown; it is interesting that widespread degenerative changes have been observed in both the hypothalamus and pituitary of sheep with scrapie (196). The forebrains from scrapie-infected mice have been added to human pituitary suspensions to determine if prions and HGH copurify (223). Bioassays in mice suggest that prions and HGH do not copurify with currently used protocols (242). Although these results seem reassuring, especially for patients treated with HGH over much of the past decade, the relatively low titers of the murine scrapie prions used in these studies may not have provided an adequate test (202). The extremely small size and charge heterogeneity exhibited by scrapie (4, 17, 154, 238, 239) and presumably CJD prions (14, 197) may complicate procedures designed to separate pituitary hormones from these slow infectious pathogens. Even though additional investigations argue for the efficacy of inactivating prions in HGH fractions prepared from human pituitaries using 6 M urea (235), it seems doubtful that such protocols will be used for purifying HGH because recombinant HGH is available.

Molecular genetic studies have shown that most patients developing iatrogenic CJD after receiving pituitary derived HGH are homozygous for either methionine or valine at codon 129 of the PrP gene (203, 209, 212). Homozygosity at the codon 129 polymorphism has also been shown to predispose individuals to sporadic CJD (234). Interestingly, valine homozygosity seems to be overrepresented in these HGH cases compared with the general population.

Five cases of CJD have occurred in women receiving human pituitary gonadotropin (207, 208, 219).

BOVINE SPONGIFORM ENCEPHALOPATHY

Since 1986, more than 150,000 cattle have died of BSE in Great Britain (189, 190). Some investigators contend that BSE resulted from feeds made of meat and bone meal prepared from rendered sheep offal. The diminished use of hydrocarbon extraction in the rendering of sheep offal may be the reason that scrapie prions survived the rendering process. Since 1988, the practice of using dietary protein supplements for domestic animals derived from rendered sheep or cattle offal has been forbidden in the United Kingdom. Whether BSE will disappear with the cessation of feeding animals rendered meat and bone meal remains to be established.

Brain extracts from BSE cattle have transmitted disease to mice, cattle, sheep, and pigs after intracerebral inoculation (24, 44, 45, 62). Transmissions to mice and sheep suggest that cattle preferentially propagate a single strain of prions. Seven BSE brains all produced similar incubation times as measured in each of three strains of inbred mice (24). Of particular importance in the BSE epidemic is the recent transmission of BSE to a nonhuman primate, the marmoset, after a prolonged incubation period (6).

SYNTHESIS OF PrP^C AND PrP^{Sc}

Metabolic labeling studies of scrapie-infected cultured cells have shown that PrP^{Sc} is synthesized slowly through a posttranslational process (18, 19, 33) in contrast to PrP^C, which is synthesized and degraded rapidly (32). Both PrP isoforms appear to pass through the Golgi apparatus, where their Asn-linked oligosaccharides are modified and sialylated (17, 57, 85, 116, 162). PrP^C is presumably transported within secretory vesicles to the external cell surface, where it is anchored by a glycosyl phosphatidylinositol (GPI) moiety (164, 174, 176). In contrast, PrP^{Sc} accumulates primarily within cells, where it is deposited in cytoplasmic vesicles, many of which appear to be secondary lysosomes (19, 34, 124, 179, 180).

Several experimental results indicate that PrP molecules destined to become PrP^{Sc} exit to the cell surface, similar to PrP^C (176), prior to their conversion into PrP^{Sc} (19, 33, 179). Interestingly, the GPI anchors of both PrP^C and PrP^{Sc} are sialylated (174). These structures presumably play a major role in directing the subcellular trafficking of these molecules. The reentry of PrP^C into cells appears to occur through the caveolae (5).

TRANSGENETICS AND GENE TARGETING

The passage of prions between species is a stochastic process characterized by prolonged incubation times (139). Prions synthesized de novo reflect the

sequence of the host PrP gene and not that of the PrP^{Sc} molecules in the inoculum (15). Upon subsequent passage in a homologous host, the incubation time shortens to that recorded for all subsequent passages and it becomes a nonstochastic process. The species barrier concept is of practical importance in assessing the risk for humans of developing CJD after consumption of scrapie-infected lamb or BSE-infected beef (151, 189).

Transgenic Mice Expressing Syrian Hamster PrP

To test the hypothesis that differences in PrP gene sequences might be responsible for the species barrier, Tg mice expressing SHaPrP were used (156, 167). The PrP genes of Syrian hamsters and mice encode proteins differing at 16 positions. Incubation times in four lines of Tg(SHaPrP) mice inoculated with Mo prions were prolonged compared with those observed for non-Tg, control mice. Inoculation of Tg(SHaPrP) mice with SHa prions demonstrated abrogation of the species barrier, resulting in abbreviated incubation times due to a nonstochastic process (156, 167). The length of the incubation time after inoculation with SHa prions was inversely proportional to the level of $SHaPrP^{C}$ in the brains of Tg(SHaPrP) mice (156). $SHaPrP^{Sc}$ levels in the brains of clinically ill mice were similar in all four Tg(SHaPrP) lines inoculated with SHa prions. Bioassays of brain extracts from clinically ill Tg(SHaPrP) mice inoculated with Mo prions revealed only Mo prions but no SHa prions. Conversely, inoculation of Tg(SHaPrP) mice with SHa prions led to the synthesis of only SHa prions. Thus, the de novo synthesis of prions is species specific and reflects the genetic origin of the inoculated prions. Similarly, the neuropathology of Tg(SHaPrP) mice is determined by the genetic origin of prion inoculum. Mo prions injected into Tg(SHaPrP) mice produced a neuropathology characteristic of mice with scrapie. A moderate degree of vacuolation in both the gray and white matter was found while amyloid plaques were rarely detected. Inoculation of Tg(SHaPrP) mice with SHa prions produced intense vacuolation of the gray matter, sparing of the white matter, and numerous SHaPrP amyloid plaques characteristic of Syrian hamsters with scrapie.

During transgenetic studies, we discovered that uninoculated older mice harboring high copy-numbers of wild-type PrP transgenes derived from Syrian hamsters, sheep, and PrP-B mice spontaneously developed truncal ataxia, hind-limb paralysis, and tremors (186). These Tg mice exhibited a profound necrotizing myopathy involving skeletal muscle, a demyelinating polyneuropathy, and focal vacuolation of the central nervous system (CNS). Development of disease depended on transgene dosage. For example, $Tg(SHaPrP^{+/+})7$ mice homozygous for the SHaPrP transgene array regularly developed disease between 300 and 500 days of age while hemizygous $Tg(SHaPrP^{+/0})7$ mice also developed disease, but only after >650 days.

Attempts to demonstrate PrP^{Sc} in either muscle or brain were unsuccessful, but transmission of disease with brain extracts from $Tg(SHaPrP^{+/+})7$ mice inoculated into Syrian hamsters did occur. These Syrian hamsters had PrP^{Sc} as detected by immunoblotting and spongiform degeneration (D Groth & SB Prusiner, unpublished data). Serial passage with brain extracts from these animals to recipients was observed. De novo synthesis of prions in $Tg(SHaPrP^{+/+})7$ mice overexpressing wild-type $SHaPrP^{C}$ supports the hypothesis that sporadic CJD does not result from infection but rather is a consequence of the spontaneous, although rare, conversion of PrP^{C} into PrP^{Sc}. Alternatively, a somatic mutation in which mutant $SHaPrP^{C}$ is spontaneously converted into PrP^{Sc} as in the inherited prion diseases could also explain sporadic CJD. These findings as well as those described below for Tg(MoPrP-P101L) mice argue that prions are devoid of foreign nucleic acid, a conclusion in accord with many earlier studies (reviewed above) that used other experimental approaches.

Artificial Prions

Transgenic mice expressing chimeric PrP genes derived from SHa and MoPrP genes were constructed (169). One SHa/MoPrP gene, designated MH2M PrP, contains five amino acid substitutions encoded by SHaPrP while another construct designated MHM2 PrP has two substitutions. Tg(MH2M PrP) mice were susceptible to both SHa and Mo prions, whereas three lines expressing MHM2 PrP were resistant to SHa prions (168). The brains of Tg(MH2M PrP) mice dying of scrapie contained chimeric PrP^{Sc} and prions with an artificial host range favoring propagation in mice that express the corresponding chimeric PrP. The prions were also transmissible, at reduced efficiency, to non-Tg mice and hamsters. These findings provide genetic evidence for homophilic interactions between PrP^{Sc} in the inoculum and PrP^{C} synthesized by the host.

Ablation of the PrP Gene in Mice

Ablation of the PrP gene in Tg ($Prn\text{-}p^{0/0}$) mice, unexpectedly, does not affect the development of these animals (28). In fact, they are healthy at almost 2 years of age. $Prn\text{-}p^{0/0}$ mice are resistant to prions and do not propagate scrapie infectivity (27, 152). Crossing these mice with Tg(SHaPrP) mice rendered them susceptible to to SHa prions, but they remained resistant to Mo prions (27, 152). Given that the absence of PrP^{C} expression does not provoke disease, scrapie and other prion diseases are probably a consequence of PrP^{Sc} accumulation rather than an inhibition of PrP^{C} function (28).

Mice heterozygous ($Prn\text{-}p^{0/+}$) for ablation of the PrP gene had prolonged incubation times when inoculated with mouse prions (152). The $Prn\text{-}p^{0/+}$ mice developed signs of neurologic dysfunction at 400–460 days after inoculation.

These findings are in accord with studies on Tg(SHaPrP) mice in which diminished incubation times accompanied increased SHaPrP expression (156).

Since Prn-$p^{0/0}$ mice do not express PrP^C, we reasoned that they might more readily produce α-PrP antibodies. Prn-$p^{0/0}$ mice immunized with Mo or SHa prion rods produced α-PrP antisera that bound Mo, SHa, and human PrP (152). These findings contrast with earlier studies in which α-MoPrP antibodies could not be produced in mice, presumably because the mice had been rendered tolerant by the presence of $MoPrP^C$ (7, 100, 161). That Prn-$p^{0/0}$ mice readily produce α-PrP antibodies is consistent with the hypothesis that the lack of an immune response in prion diseases stems from the fact that PrP^C and PrP^{Sc} share many epitopes. Whether Prn-$p^{0/0}$ mice produce α-PrP antibodies that specifically recognize conformation-dependent epitopes present on PrP^{Sc} but absent from PrP^C remains to be determined.

Transgenic Mice Expressing Mutant PrP

The codon 102 point mutation found in GSS patients was introduced into the MoPrP gene, and Tg(MoPrP-P101L)H mice were created expressing high (H) levels of the mutant transgene product. The Tg(MoPrP-P101L)H mice spontaneously developed CNS degeneration, characterized by clinical signs indistinguishable from experimental murine scrapie and neuropathology consisting of widespread spongiform morphology and astrocytic gliosis (93) as well as PrP amyloid plaques. By inference, these results contend that PrP gene mutations cause GSS, familial CJD, and FFI.

Brain extracts prepared from Tg(MoPrP-P101L)H mice transmitted CNS degeneration to Tg196 mice expressing low levels of the mutant transgene product. In addition, some Syrian hamsters developed CNS degeneration between 115 and 600 days after inoculation (247). Recent studies have demonstrated serial transmission of neurodegeneration from inoculated Tg(MoPrP-P101L)H mice to inoculated Tg196 mice. Undetectable or low levels of PrP^{Sc} in the brains of the Tg(MoPrP-P101L)H mice are consistent with the results of these transmission experiments, which suggest low titers of infectious prions. Although immunoassays conducted after limited proteolysis detected no PrP^{Sc} in the brains of inoculated Tg196 mice exhibiting neurologic dysfunction, PrP amyloid plaques were often found. The neurodegeneration found in inoculated Tg196 mice likely results from a modification of mutant PrP^C that is initiated by mutant PrP^{Sc} present in the brain extracts prepared from ill Tg(MoPrP-P101L)H mice. In support of this explanation are the findings for some inherited human prion diseases in which neither protease-resistant PrP (23, 126) nor transmission to experimental animals could be demonstrated (181). Furthermore, transmission of disease from Tg(MoPrP-P101L)H mice to Tg196 mice but not to Swiss mice is consistent with earlier findings demonstrating that homotypic interactions between PrP^C and PrP^{Sc} act in the formation of PrP^{Sc}, as described above.

PRION PROPAGATION

Although the search for a scrapie-specific nucleic acid continues without success, some investigators steadfastly cling to the notion that this putative polynucleotide drives prion replication. If prions are found to contain a scrapie-specific nucleic acid, then such a molecule would be expected to direct scrapie agent replication using a strategy similar to that employed by viruses. In absence of any chemical or physical evidence for a scrapie-specific polynucleotide, it seems reasonable to consider some alternative mechanisms that might play major roles in prion biosynthesis. The multiplication of prion infectivity is an exponential process in which the posttranslational conversion of PrP^C or a precursor to PrP^{Sc} appears to be obligatory (18).

Propagation of Prions Involves Formation of a Homotypic PrP^C-PrP^{Sc} Complex

PrP^{Sc} appears to combine with PrP^C to form a PrP^C-PrP^{Sc} complex, which is subsequently transformed into two molecules of PrP^{Sc}. In the next cycle, two PrP^{Sc} molecules combine with two PrP^C molecules. The resulting two complexes then dissociate to combine with four PrP^C molecules, creating an exponential process. Studies with Tg(SHaPrP) mice argue that prion synthesis involves replication, not merely amplification (156). Assuming prion biosynthesis simply involves amplification of posttranslationally altered PrP molecules, we might expect Tg(SHaPrP) mice to produce both SHa and Mo prions after inoculation with either prion because these mice produce both SHa and $MoPrP^C$. Yet Tg(SHaPrP) mice synthesize only those prions present in the inoculum. These results suggest that the incoming prion containing PrP^{Sc} interacts with the homotypic PrP^C substrate to replicate more of the same prions.

Conformational Changes During Conversion of PrP^C into PrP^{Sc}

Since studies of PrP^{Sc} failed to reveal a candidate posttranslational chemical modification that might distinguish it from PrP^C (175), we considered the possibility that these two PrP isoforms may differ only in their conformations. To assess this possibility, Pan et al (138) determined the secondary structures of PrP^C and PrP^{Sc}. Fourier transform infrared (FTIR) spectroscopy demonstrated that PrP^C has a high α-helix and low β-sheet content, findings confirmed by circular dichroism measurements (138). In contrast, PrP^{Sc} contained more than 40% β-sheet and 30% α-helix as measured by FTIR. The N-terminally truncated PrP^{Sc} derived by limited proteolysis and designated PrP 27–30

showed an even higher β-sheet and a lower α-helix content than that found for PrP^{Sc} (35, 70). Although these findings argue that the conversion of α-helices into β-sheets underlies the formation of PrP^{Sc}, we cannot eliminate the possibility that an undetected chemical modification of a small fraction of PrP^{Sc} initiates this process.

Structure prediction studies of $SHaPrP^{C}$ and $SHaPrP^{Sc}$ (residues 23–231) were performed using a neural network algorithm (107, 145). Class-dependent (α/α, α/β, β/β) and naive predictions were performed. The α/α class contains proteins composed largely of α-helices. Similarly, the β/β class contains proteins that are mostly β-sheets. Interestingly, the four putative α-helical domains of PrP (71) showed both strong helix preference in the α/α class prediction and strong β-sheet preference in the β/β class prediction. These results are consistent with the hypothesis that these domains undergo conformational changes from α-helices to β-sheets during the formation of PrP^{Sc}. Further support for this hypothesis comes from structural investigations of synthetic PrP peptides.

Secondary Structures of PrP Synthetic Peptides

Three of the four peptides corresponding to the four putative α-helical domains of PrP^{C} formed amyloid polymers with high β-sheet content when dispersed into water but formed α-helices in hexafluoroisopropanol (71). Furthermore, denaturation of PrP 27–30 under conditions that reduced scrapie infectivity concomitantly diminished the β-sheet content (70). Thus, both the conversion of PrP^{C} to PrP^{Sc} and the propagation of infectious prion particles probably involve a structural transition in which α-helical domains acquire β-sheets.

In humans carrying point mutations or inserts in their PrP genes, mutant PrP^{C} molecules might spontaneously convert into PrP^{Sc}. While the initial stochastic event may be inefficient, once it happens the process becomes autocatalytic. The proposed mechanism is consistent with observations made from individuals harboring germline mutations who do not develop CNS dysfunction for decades, and with studies on Tg(MoPrP-P101L)H mice that spontaneously develop CNS degeneration (93). Whether all GSS and familial CJD cases contain infectious prions or some represent inborn errors of PrP metabolism in which neither PrP^{Sc} nor prion infectivity accumulates is unknown; however, transmission of inherited human prion diseases to animals is less frequent than transmission of sporadic CJD (181). Therefore, mutant PrP^{C} molecules alone can probably also produce CNS degeneration.

PRION DIVERSITY

The diversity of scrapie prions was first appreciated in goats inoculated with hyper and drowsy isolates (141). Subsequently, studies in mice demonstrated the existence of many scrapie strains (25, 48, 50, 102), an observation that

continues to pose a fascinating conundrum. What is the macromolecule that carries the information required for each strain to manifest a unique set of biological properties if it is not a nucleic acid?

There is good evidence for multiple "strains" or distinct isolates of prions as defined by specific incubation times, distribution of vacuolar lesions, and patterns of PrP^{Sc} accumulation (26, 49, 61, 87). Incubation times have been used to distinguish strains inoculated into sheep, goats, mice, and hamsters. Recent studies [e.g. with Tg(SHaPrP) mice (47, 152) and with mice expressing chimeric Mo/SHaPrP transgenes (168)] have shown that the incubation time is not characteristic of a particular strain but rather depends on the host. For example, three SHa prion strains passaged in Syrian hamsters (102, 103) had profoundly different incubation times depending upon the host in which they were passaged.

For many years some investigators have argued that scrapie is caused by a virus-like particle that contains a scrapie-specific nucleic acid encoding the information expressed by each isolate (25). To date, no such polynucleotide has been identified, although various techniques including measurements of the nucleic acids in purified preparations have been used. An alternative hypothesis is that PrP^{Sc} alone is capable of transmitting disease but its characteristics might be modified by a cellular RNA (185). This accessory cellular RNA is postulated to induce its own synthesis upon transmission from one host to another. However, recent studies with two prion strains demonstrate similar levels of resistance to inactivation by UV irradiation, which argues convincingly against the cellular RNA hypothesis (SB Prusiner, A Serban & J Cleaver, unpublished data).

The finding that the pattern of PrP^{Sc} accumulation in the CNS is characteristic for a particular strain offers a mechanism for the propagation of distinct prion isolates (87). In this model, a different set of cells would propagate each isolate. Whether different Asn-linked CHOs target PrP^{Sc} of a distinct isolate to a particular set of cells expressing specific surface lectins that function as receptors remains to be established (87). These surface lectins would bind the same Asn-linked CHOs that are covalently attached to PrP^{C} during its synthesis and remain bound during the conversion of PrP^{C} into PrP^{Sc}. The great diversity of Asn-linked CHOs makes them potential candidates for carrying isolate-specific information (147). Even though this hypothesis is attractive, one should note that PrP^{Sc} synthesis in scrapie-infected cells occurs in the presence of tunicamycin, which inhibits Asn-linked glycosylation, and with PrP molecules mutated at the Asn-linked glycosylation consensus sites (180). Although the structures of Asn-linked CHOs have been analyzed for PrP^{Sc} of one isolate (57), no data are available for PrP^{Sc} of other isolates or PrP^{C}. The large number of Asn-linked CHOs found attached to the PrP 27–30 of Sc237 prions purified from Syrian hamster would seem to refute the argument for Asn-linked CHOs

being responsible for strain variation, but experimental data addressing this point are still needed.

Another possible explanation for the region-specific distribution of PrP^{Sc} in brain observed in each strain involves the formation of a complex between PrP^{Sc} and an as-yet-undetected peptide or protein of cellular origin. Such a complex would bind cell-specific receptors, thus facilitating the entry of PrP^{Sc} into those cells. Contradicting this hypothesis is the finding that the properties of SHa(Sc237) and Mo(RML) prions do not change upon passage through the spleen. Furthermore, no auxilliary proteins have been found to purify with PrP^{Sc}. In favor of such a hypothesis is the fact that receptors for proteins are numerous and could provide the specificity required. Whether either of the two peptides consistently found in purified preparations of Sc237 prions (175) targets prions to specific cells remains to be determined.

Alternatively, explaining the problem of multiple distinct prion isolates might be accommodated by multiple PrP^{Sc} conformers that act as templates for the folding of de novo synthesized PrP^{Sc} molecules during prion replication. A conformer corresponding to a specific strain would need to bind to a particular PrP receptor, which would either facilitate its entry into cells or the conversion of PrP^{C} into PrP^{Sc} in those particular cells. Although all these proposals are rather unorthodox, they are consistent with observations generated from Tg(SHaPrP)Mo studies contending that PrP^{Sc} in the inoculum binds to homotypic PrP^{C} to form an intermediate in the propagation of prions (156). Whether foldases, chaperonins, or other types of molecules participate in the conversion of the PrP^{C}/PrP^{Sc} complex into two molecules of PrP^{Sc} is unknown. The molecular weight of a PrP^{Sc} homodimer is consistent with the ionizing radiation target size of 55,000 ± 9000 Daltons as determined for infectious prion particles independent of their polymeric form (9). Of note, two different isolates from mink dying of transmissible mink encephalopathy exhibit different sensitivities of PrP^{Sc} to proteolytic digestion, supporting the suggestion that isolate-specific information might be carried by PrP^{Sc} (12, 13, 117).

SOME CONCLUDING REMARKS AND A PERSPECTIVE

The study of prions has taken several unexpected paths over the past few years. The discovery that prion diseases in humans are uniquely both genetic and infectious has greatly strengthened and extended the prion concept. To date, 18 different mutations in the human PrP gene all resulting in nonconservative substitutions have been found to be either linked genetically to or segregate with the inherited prion diseases (Figure 1). Yet the transmissible prion particle is composed largely, if not entirely, of an abnormal isoform of the prion protein designated PrP^{Sc} (148). These findings argue that prion diseases should be considered pseudoinfections because the particles transmitting disease appear

to be devoid of a foreign nucleic acid and thus differ from all known microorganisms as well as viruses and viroids. Because much information, especially about scrapie of rodents, has been derived using experimental protocols adapted from virology, we continue to use terms such as infection, incubation period, transmissibility, and endpoint titration in studies of prion diseases.

Transgenic mice expressing foreign or mutant PrP genes now permit virtually all facets of prion diseases to be studied and have created a framework for future investigations. Furthermore, the structure and organization of the PrP gene suggested that PrP^{Sc} is derived from PrP^{C} or a precursor by a posttranslational process. Studies with scrapie-infected cultured cells have provided much evidence that the conversion of PrP^{C} to PrP^{Sc} is a posttranslational process that probably occurs in the endocytic pathway. The molecular mechanism of PrP^{Sc} formation remains to be elucidated, but chemical and physical studies have shown that the conformations of PrP^{C} and PrP^{Sc} are profoundly different.

The study of prion biology and diseases is a new and emerging area of biomedical investigation. Prion biology has its roots in virology, neurology, and neuropathology, but its relationships to the disciplines of molecular and cell biology as well as protein chemistry have become evident only recently. Certainly, learning how prions multiply and cause disease will open up new vistas in biochemistry and genetics.

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